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Metal exposure and accumulation patterns in free-range cows (*Bos taurus*) in a contaminated natural area: Influence of spatial and social behavior

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ABSTRACT

Possible effects of spatial metal distribution, seasonal-, ecological- and ethological parameters, on the metal exposure of cows were investigated. Therefore the habitat use, vegetation selection and foraging behavior of two free ranging Galloway herds in a metal polluted nature reserve were observed. Metal concentrations in soil, vegetation, hair, blood and feces were measured. Although both herds lived in the same reserve, their metal exposure differed significantly. A high consumption of soft rush by herd 1 during winter for instance was responsible for a large increase in daily Cd intake. The results of this study suggest that the exposure and health risks of large grazers can probably not only be predicted by a general monitoring of soil and vegetation pollution. Also detailed information about the occurring vegetation types, spatial habitat use together with the social- and foraging behavior and diet selection of the species need to be studied.

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1. Introduction

As a result of the widespread metal contamination, organisms often spent their life in metal polluted areas. Grazers are exposed to these metals via ingestion of polluted vegetation, small amounts of soil and in some cases also via drinking water (Reglero et al., 2008). Because large grazers are often exposed to polluted herbages and soil, they are used in several studies as bio indicators for metal pollution. In such studies non-destructive samples of the animals may be used such as blood, milk and hair (Patrashkov et al., 2003; Rashed and Soltan, 2005; Somasundaram et al., 2005), but also destructive samples such as liver, kidney and muscle (Alonso et al., 2002b; Cai et al., 2009; Kramarova et al., 2005; Lusky et al., 1992; Mor et al., 2005; Roberts et al., 1994). In addition, models have been developed to asses the risks of metals to wildlife. These models are used to predict the risk for an organism, based on concentrations in soil and sometimes vegetation (Van den Brink et al., 2010). Also other models have been developed to predict accumulation of metals in body tissues and milk of ruminants used for human consumption (Beresford et al., 1999; Crout et al., 2004; Phillips and Tudoreanu, 2011). The accuracy of these prediction models depends on the spatial behavior and the diet of the organism. The smaller the range and the simpler the diet of the organisms, the more accurate predictions based on environmental concentrations may be (Van den Brink et al., 2011). Large grazers range at a larger scale than small herbivores e.g. mice, voles, rabbits and squirrels and integrate metals from a larger area and most likely also from different vegetation types. Therefore other factors are more important in predicting exposure. Some studies suggest that metal accumulation in grazers is also influenced by variation in climatic conditions, season and herbage growth (Massanyi et al., 2003; Rhind et al., 2005). For grazer species such as cattle, their social and spatial behavior may need to be taken into account as well. For that reason the soil–plant–animal relationships were studied in this case study with a focus on the ecological and ethological characteristics of cows. The first step was the investigation of the soil–plant relationships and metal uptake into vegetation that was eaten by the cows. Therefore the bioavailability and accumulation of metals in vegetation need to be taken into account. These parameters are controlled by the adsorption capacity of the soil. This depends on soil properties like pH, organic matter content, cation exchange capacity (CEC), oxidation–reduction status (Eh), clay fraction, calcium carbonate and Fe and Mn oxides (Fanrong et al., 2011; Malandrino et al., 2011; Yusuf et al., 2011). Among these soil properties, pH and organic matter content are generally considered to play the most important role in determining the bioavailability of metals (Tudoreanu and Phillips, 2004a, 2004b, Fanrong et al., 2011).

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Plants accumulate most metals via the soil–root pathway but some metals can also enter plants via leaves or will be externally adsorbed to vegetation via the air–plant pathway by atmospheric deposition (Schreck et al., 2012). Both internal and external metals from plants are available for grazers (Reglero et al., 2008; Yusuf et al., 2011). When also metal intake via the air–lung pathway is considered, this could imply that predictions only based on soil data may underestimate the potential exposure risk, especially when the source of pollution is still active.

The most important metal exposure route for grazers is their food (Bruce et al., 2003; van der Fels-Klerx et al., 2011). Cattle, especially females, always graze in a herd and perform a high synchrony of behavior (Sárová et al., 2007). Therefore, individuals of the same herd will encounter the same available vegetation and may thus have more or less the same diet and therefore also a comparable metal uptake. Cattle are ruminants and spend approximately 5–7 h/d eating, 7–10 h/d ruminating, 30 min/d drinking and require approximately 10 h/d of lying and (or) resting time (Grant and Albright, 2001). When the exposure of cattle is studied based on their vegetation and habitat use, their habitat specific behavior is very important to take into account because some habitats are probably used only for sleeping, drinking or ruminating. When herbivores are grazing in a heterogeneous habitat, it is likely that their feeding pattern will also be heterogeneous (Wallis de Vries and Daleboudt, 1994). A heterogeneous habitat implies a heterogeneous spatial distribution of the available metals to grazers. In addition, seasonal variation in weather conditions and herbage growth will have an effect on the bioavailability of metals and therefore on the exposure for grazers. Knowledge of these parameters, together with the understanding of the grazer's diet selecting behavior is therefore important to allow effective prediction of their exposure risk (Fritsch et al., 2011). Daily ingestion of low metal concentrations will not lead to acute poisoning symptoms for grazers but in case metals are being retained in the body, their concentrations may rise during the life span of the cattle (10–20 years). As a result, the risks for health problems may increase with age. In general, non-essential metals like Cd, Pb and As can cause mutagenicity, carcinogenicity, teratogenicity, immunosuppression, poor body condition and impaired reproduction (Alonso et al., 2002a). In addition to these general effects, each metal has its own specific health effects. For example lead intake can affect the gastrointestinal, renal, nervous and hemopoietic systems (Swarup et al., 2006). Because cattle are consumed by humans, chronic metal exposure of the cows may also pose a human health risk (Wilkinson et al., 2003; Prankel et al., 2005). Therefore, it is important to investigate the impact of ecological and behavioral aspects on the exposure of metals grazers.

The aim of this case-study was to investigate the effect of spatial metal distribution, soil characteristics, vegetation type, annual variation, habitat use and diet selection in a terrestrial ecosystem, on the exposure and potential risk of metals to cattle. Different types of data were collected to provide an accurate insight in all possible mechanisms and relationships that may influence metal exposure of grazers. Because previous studies mainly focused on soil and vegetation accumulation data, this study will provide new insights in the role of other ecological and ethological mechanisms, likely underestimated in the past.

2. Materials and methods

2.1. Study area and animals

The nature reserve "Hageven-Plateaux" was selected as study area. This reserve is located on the border of Belgium and the Netherlands (Fig. 1). It is a 555 ha large

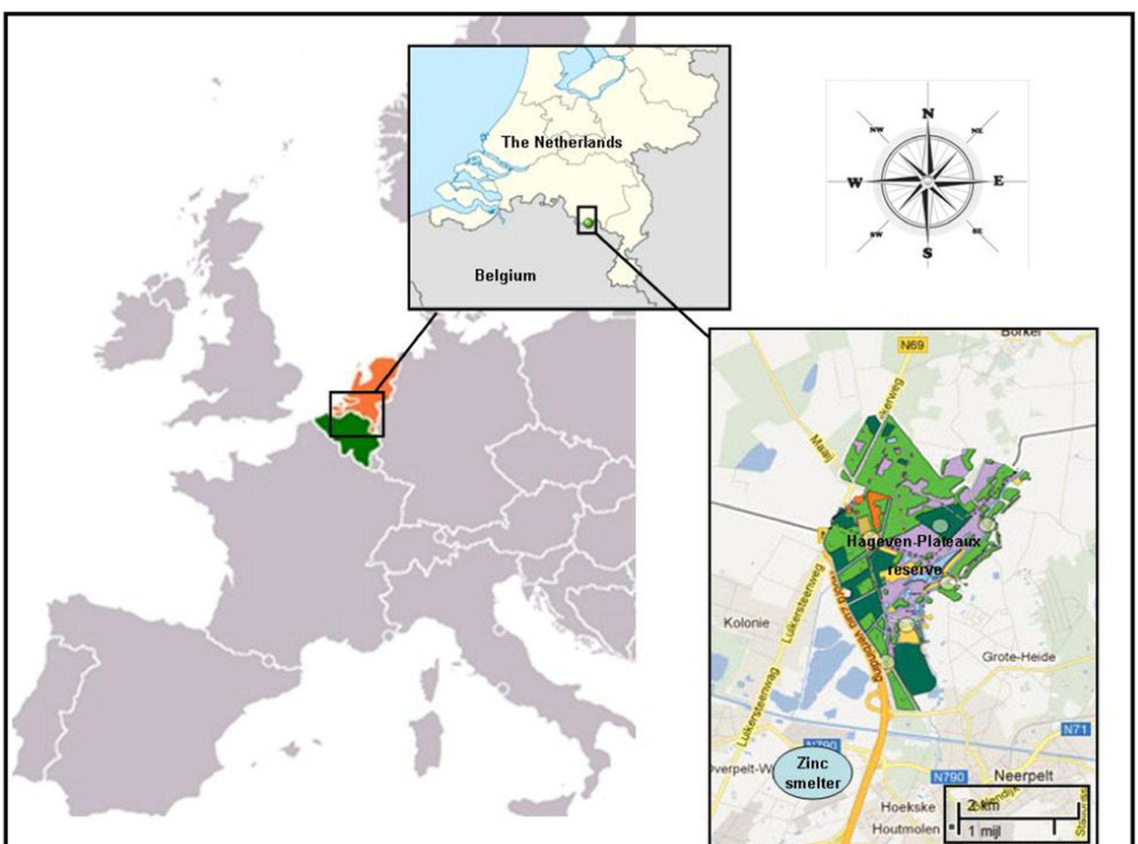

Fig. 1. Map of the location of the Hageven-Plateaux reserve and the Zinc smelter.

reserve existing of heath land, ponds and forests and is situated at 1.5 km north of a zinc smelter which caused metal pollution in the region during the second half of last century (Nawrot et al., 2006). In this reserve 42 mostly female cattle (*Bos taurus*) of the Galloway breed, lived year-round as ecological engineers to create a diverse vegetation pattern and manage the heath land. The study animals were not domesticated by humans, free ranging and therefore lived in semi-wild conditions, without additional feeding. The age of the cows ranged from 2 to 15 years old and they all resided their whole live in that reserve from birth.

2.2. Field studies

Data of two different field studies conducted in the same reserve were combined.

Field study 1 was conducted during spring and summer of 2008. In April 2008 blood and hair samples of the Galloways were collected. During July and August 2008 soil and corresponding vegetation samples were collected. The aim of field study 1 was to generally monitor the metal pollution in the reserve with a focus on the soil–plant relationship.

Field study 2 was an ecological-exposure study, conducted during summer and winter of 2009–2010. During July, August and September 2009, summer foraging behavior and habitat use of the Galloways was observed and vegetation and fecal samples of the Galloways were collected. During December 2009 and January–February 2010, winter foraging behavior and habitat use of the Galloways was observed and vegetation samples were collected. The aim of field study 2 was to investigate the metal exposure of the Galloway cows with a focus on the plant–grazer relationship.

2.3. Field study 1

2.3.1. Soil and corresponding vegetation

During field study 1 in total 155 sampling spots were selected based on an objective grid (stratified random sampling), using ArcGis software (see Figs. S1 and S2). In each square of the grid of 40,000 m^2, a soil and vegetation sample was taken randomly. To locate the sampling spots in the field a 'Garmin foretrex 201 personal navigator' GPS was used. This allowed to obtain a non-biased insight in the spatial differences in metal pollution of the whole reserve. This grid method is often used by governments for monitoring purposes (Theocharopoulos et al., 2001).

Vegetation ($\pm$400 cm^2) was collected by cutting with stainless steel scissors from 1 cm above the soil. No classification of the different vegetation types was made. One liter of soil was collected over 10 cm of depth after removal of the upper organic layer and stored in a plastic bucket using a stainless steel shovel. Soil samples were sieved over a 2 mm sieve, divided in 4 subsamples and stored in 50 ml falcon tubes. These subsamples were used for the analysis of metals, pH, clay fraction and organic mater. The soil samples for metal analysis were dried at room temperature for 96 h. Vegetation samples were oven dried at 60° for 96 h. Both soil and vegetation samples were stored at room temperature in closed polypropylene tubes until metal analysis.

2.3.2. Hair and blood samples

To collect blood and hair samples during field study 1, all Galloway cows were captured together (using hay to lure them) with the help of the nature managers from the reserve. Each blood sample was taken at the tail, by a veterinarian. Before the blood sample started clotting, 1 ml blood was immediately pipetted in a pre-weighed 14 ml falcon tube. Back at the laboratory the 1 ml samples were weighed and stored in a freezer at −20 °C until analysis. Immediately after the collection of the blood sample, a hair sample ($\pm$5 cm long) was taken from the coat at the side of the neck with stainless steel scissors. The samples were put in plastic bags and stored in a freezer (−20 °C) until analysis.

2.4. Field study 2

2.4.1. Measuring spatial habitat and vegetation use of the Galloways

To measure the habitat and vegetation use of the cows an observation protocol was designed, based on the time sampling method (more specific: "main activity sampling") to observe animal behavior (Altmann, 1974; Martin and Bateson, 2007). During summer (July–August–September) and winter (December–January–February) the Galloways were followed on foot and observed during day and night. In total the cows were observed 105 h spread over 15 days in summer and 120 h spread over 17 days in winter. During the whole study period, the Galloway cows had spontaneously split up in 2 herds. Every herd was at least one day followed during morning, afternoon, evening or night and these observation periods were chosen *at random*. The animal composition of each herd was noted at the beginning of each observation. In the beginning of the observations a habituation period of one day was conducted to let the animals get used to the observer and to avoid an influence on their grazing behavior. Parts of the reserve where the cows mainly went for resting or drinking were excluded from the observations for their vegetation use. This exclusion was done by scoring grazing behavior only when more than 50% of the herd was grazing (also called a grazing bout). During a grazing bout a vegetation sample was taken every 15 min. The sampling location was selected based on the position in the middle of the herd and the visually observed vegetation selection of the animals. Only the vegetation that effectively was eaten by the animals was collected by cutting with stainless steel scissors till 1 cm above the ground and put in a paper bag. From consumed trees or bushes a branch was cut of from approximately 1 m above the ground. Roots of the plants were not collected because these parts of the plants are irrelevant based on the way cattle grazes: they grab the plants with their tongue, press them to the hard upper pad and tear it off a few cm above the ground (Hall and Silver, 2009). This way they generally don't ingest the roots of the plants. From every sampling location the coordinates were measured using a 'Garmin foretrex 201 personal navigator' GPS. From every sampling location the vegetation type(s) were determined: heath (*Calluna vulgaris*), grass (poaceae), birch (*Betula* spp.), oak (*Quercus* spp.) soft rush (*Juncus effusus*) and bush. Also the date and time of the sampling were registered.

2.4.2. Collection of fecal samples

During summer fecal samples were collected. Both herds were followed and when an animal started defecating, the identity of the animal was registered by reading the sanitel identification number on their ear-label, using a binocular. The fecal samples were taken using a plastic bag, avoiding contamination with soil or vegetation. Of each sample the time, date and cow number was registered. In the lab all samples were frozen (−20 °C) till analysis.

2.5. Sample preparation and analysis

2.5.1. Metal analysis

From each soil sample between 0.5 and 1.0 g of dried soil was accurately weighed in Teflon® vessels and a mixture of HNO_3 (69%) and HCl (37%) (1:3, *vv*) was added. Subsequently, samples were transferred to Teflon® bombs and digested in a microwave oven (Tessier et al., 1984). Microwave digestion (Ethos 900, Milestone, Shelton, CT, USA) was completed in four successive steps (5 min at 90, 200, 350 and 500 W respectively).

All blood samples were freeze dried in the lab and reweighed followed by digestion for metal analysis.

All hair samples ($\pm$0.05 g) were washed with acetone during 5 min to remove external contamination. Subsequently the samples were rinsed 3 times with Milli-Q water (Millipore®, Brussels, Belgium), oven dried at 60 °C for 96 h and digested for metal analysis.

Each fecal sample was weighed and oven dried at 60 °C for 48 h, reweighed and digested for metal analysis.

Hair, blood and feces were digested with nitric acid (69%, Pro analysis, Merck) and hydrogen peroxide (30%, Pro analysis, Merck) followed by open microwave digestion (Blust et al., 1988). For the vegetation samples the same procedure was followed but HNO_3:HCl (3:1 *vv*) was used. Before measurement, all samples were diluted with Milli-Q water (Millipore, Bedford, MA, USA) up to 3–6% acid. An internal standard yttrium solution was spiked in all samples to correct for possible matrix effects. The As, Cd, Co, Cu, Pb and Zn concentrations were measured using an inductively coupled plasma–mass spectrometer (ICP–MS, model 810, Varian Inc. Australia). All metal concentrations were calculated on a dry weight basis. The accuracy of all metal analysis was verified using certified reference materials (Olive Leaves BCR-062, Skim Milk Powder BCR 063R, Human hair BCR 397, Bovine Blood ERM-CE196 and Calcareous Loam Soil CRM-141R) of the Community Bureau of Reference (Brussels, Belgium). The detection limits for Cd, Pb, Co, Cu, As and Zn were 0.2, 0.3, 0.2, 0.3, 20 and 5 ng/l respectively. At least two blanks and two reference samples were included for each batch of 40 tissue samples (Table S6). When results of the measurements were below the method of quantification limit, the detection limit of the ICP–MS was used to calculate the concentration.

2.5.2. Soil parameters

Soil pH was determined directly after sampling with a combined glass electrode (744 Metrohm, Switzerland) in a 1:5 *vv* suspension of soil and KCl (1M). Particle size distribution for the assessment of the clay fraction was analyzed via laser diffraction (Malvern Mastersizer S., Worcestershire, U.K.) (Queralt et al., 1999). The organic matter content of the soil was determined through loss on ignition (LOI). For this purpose, dry sediment was incinerated at 550 °C for 4 h (Heiri et al., 2001) The weight loss should then be proportional to the amount of organic carbon contained in the sample.

2.6. Calculation of the daily metal intake

To calculate the mean daily metal intake (DI) for a Galloway cow of the Hageven-Plateaux reserve, Equation (1) was used. In this equation the proportion of the consumed vegetation types per herd was used. This equation was based on an average body weight of a Galloway cow of 550 kg (an average Galloway bull weighs 800 kg) (Briggs and Briggs, 1980; Felius, 1996) and an average dry matter intake (DMI) per day of 2–2.5% of the body weight for cattle in general (Hicks et al., 1990; Johnson et al., 2003). This implied that Galloway cows eat on average 12.5 kg dry matter per day.

$$\mathrm{DI}_{(m)} = 12.5\ \mathrm{kg}^* \sum (P^*[m])_v \tag{1}$$

$[m]$ = mean metal concentration in μg/g dw
P = Proportion of each vegetation type in the diet
v = vegetation type.

2.7. Statistical analysis

All statistical analyses were done using the SAS institute 9.2 software. Before starting, all data were tested for normality using the Shapiro–Wilk test. The confidence interval of 95% was used to evaluate all tests.

A multiple regression test was used to determine possible linear relationships between the metal concentration in vegetation and soil, when the soil characteristics (pH, clay fraction, organic matter) were taken into account. For this analysis all parameters, except pH, were Log10 transformed.

A Fisher exact test was used to determine whether there was a significant difference in vegetation use between winter and summer and between the 2 herds. The GLIMMIX procedure with a correction for a Poisson distribution was used to determine if there was an interaction between herd and season for the vegetation use of the Galloways.

An analysis of variance (ANOVA) was used to test if the metal concentrations differed among the different vegetation types, followed by a post hoc Tukey test. Because of the low number of bush-, oak- and birch samples these 3 vegetation types were grouped together as "woody plants" for this statistical test.

A Two-way ANOVA with Log10-transformed data, followed by a post hoc Tukey test, was used to test the effect of herd and season on ingested metal via vegetation.

A spearman correlation test was used to test possible correlations between metal concentrations in the vegetation that was eaten and defecated. To do that the mean metal concentrations in vegetation eaten at day 1 were correlated with the mean metal concentrations in feces on day 2 and/or 3 because the retention time of food in cows generally lies between 48 and 72 h (Campling et al., 1963; Obitsu et al., 2009; Peyraud et al., 1989; Trinacty et al., 1999).

A *t*-test or Mann–whitney *U*-test was used to measure possible differences between the two herds for metal concentrations in blood, hair and feces.

3. Results and discussion

3.1. Field study 1

3.1.1. Soil and vegetation

The soil and vegetation samples, collected during field study 1 were obtained to provide a non-biased spatially explicit assessment of the pollution status of the whole reserve. When mapped, the results show clear variation in the spatial distribution pattern between the different metals (Figs. 2, S1 and S2). The mean soil metal concentrations were not very high but at some sampling points high concentrations were measured for Cd, Pb, As and Zn (Table S1). For Cd 23% of the soil samples exceeded the Flemish soil quality criteria of 2 μg/g for nature reserves (VLAREBO, 2008). For As, Cu, Pb and Zn less than 10% of the samples exceeded these quality standards (Table S2). For Co no standards exist. Considering these standards, the study area is classified as "moderately polluted".

When the metal concentrations in soil are compared on a map with their corresponding metal concentrations in vegetation, differential spatial variation between soil and vegetation concentrations is visible (Fig. 2). The mean metal concentrations in the vegetation samples are much lower than in their corresponding soil sample, except for Cd and Zn (Table S1). This suggests that risk assessment for large herbivores, only based on soil metal concentrations, without any information about spatial foraging behavior is probably not sufficient.

3.1.2. Relationship between metal levels in soil and vegetation

Based on the results of the multiple regression analysis of the measured parameters in soil and vegetation (all different vegetation types combined) from field study 1, the following regression model equation and Table 1 can be used to describe the relationship between metals in soil and vegetation:

$$\mathrm{Log}(\mathrm{MC}_{\mathrm{vegetation}}) = a + b \times \log(\mathrm{MC}_{\mathrm{soil}}) + c \times \mathrm{pH} + d \times \log(\mathrm{OM}) + e \times \log(\mathrm{clay}) \tag{2}$$

$\mathrm{MC}_{\mathrm{vegetation}}$ = metal concentration in vegetation (μg/g d.w.)
$\mathrm{MC}_{\mathrm{soil}}$ = metal concentration in soil (μg/g d.w.)
OM = organic matter (%)
Clay = Clay fraction (%).

For Pb, As and Cu the values of the coefficient of multiple determination (R^2) were below 0.1 and therefore these results are not shown. The other R^2 values are also quite low compared to earlier studies (de Vries et al., 2008; Römkens et al., 2009; Van Wezel et al., 2003). This is probably due to the variation in vegetation types, the importance of atmospheric deposition or the fact that only the above-ground plant tissue and not the roots were collected. Some studies already found that metal concentrations in the roots are higher than in the shoots of plants (Ping et al., 2009; Smith et al., 2010). Nevertheless, this model suggests that the lower pH and organic matter content of soil are, the higher the metal content in the vegetation will be. This is similar to earlier studies who have documented the negative correlation between soil pH and metal mobility and availability to plants (Römkens et al., 2009; Sukreeyapongse et al., 2002; Van Wezel et al., 2003; Wang et al., 2003). Other studies showed that dissolved organic matter in soils can increase the mobility and uptake of metals to plant roots (Du Laing et al., 2009; Impellitteri et al., 2002).

These results suggest that risk assessment models for grazers can not only be based on soil data. Species specific accumulation modeling, based on knowledge of the occurring vegetation types is also necessary. This can be an obstacle because in general, governments and research institutes worldwide have more data available of soil contamination than they have of contamination of plants. Predictions of the metal exposure of cattle need to focus more on the vegetation–animal relationship and less on the soil–plant relationship.

3.2. Field study 2

3.2.1. Metal concentrations in the different vegetation types

The metal concentrations were significantly different among the different vegetation types except for Cu (Fig. 3, Table S3). The mean Cd concentration in soft rush (18.4 ± 1.95 μg/g dw) was much higher than in the other vegetation types. The mean Pb concentration was the highest in grass, followed by heath and soft rush. For As the accumulation pattern was quite similar to Pb. Co levels were the highest in woody plants (trees and bush) and grass and the lowest in heath and soft rush. Zn levels were the highest in soft rush while Cu was more equally spread over the different vegetation types. Especially for the non-essential metals the difference between the different vegetation types were quite large. These results show that each metal has a different accumulation pattern in plants. This can partially be explained by the differences in soil metal concentrations and characteristics at specific locations. Some plants are growing rather on humid soils whereas others prefer dry soils. Humidity of the soil directly influences the pH which means also the bioavailability of metals (Misra and Tyler, 1999; Ahmad et al., 2011). Secondly, every plant species may have its own characteristics. Some plants accumulate metals in their roots others accumulate the most in their leaves. Previous studies already showed that plant species differ widely in their ability to absorb, accumulate and tolerate metals (Hou et al., 2011; Parkpian et al., 2003; Rascio and Navari-Izzo, 2011; Phillips and Tudoreanu, 2011; Yusuf et al., 2011; Murtaza et al., 2012). This result is similar to other habitat type-based studies with invertebrates (Vermeulen et al., 2009) and vertebrates (Fritsch et al., 2011).

When the metal concentrations in grass, heath and soft rush were compared with levels in grass and forage of other studies in Pakistan (Murtaza et al., 2012), Kenya (Jumba et al., 2007), Thailand

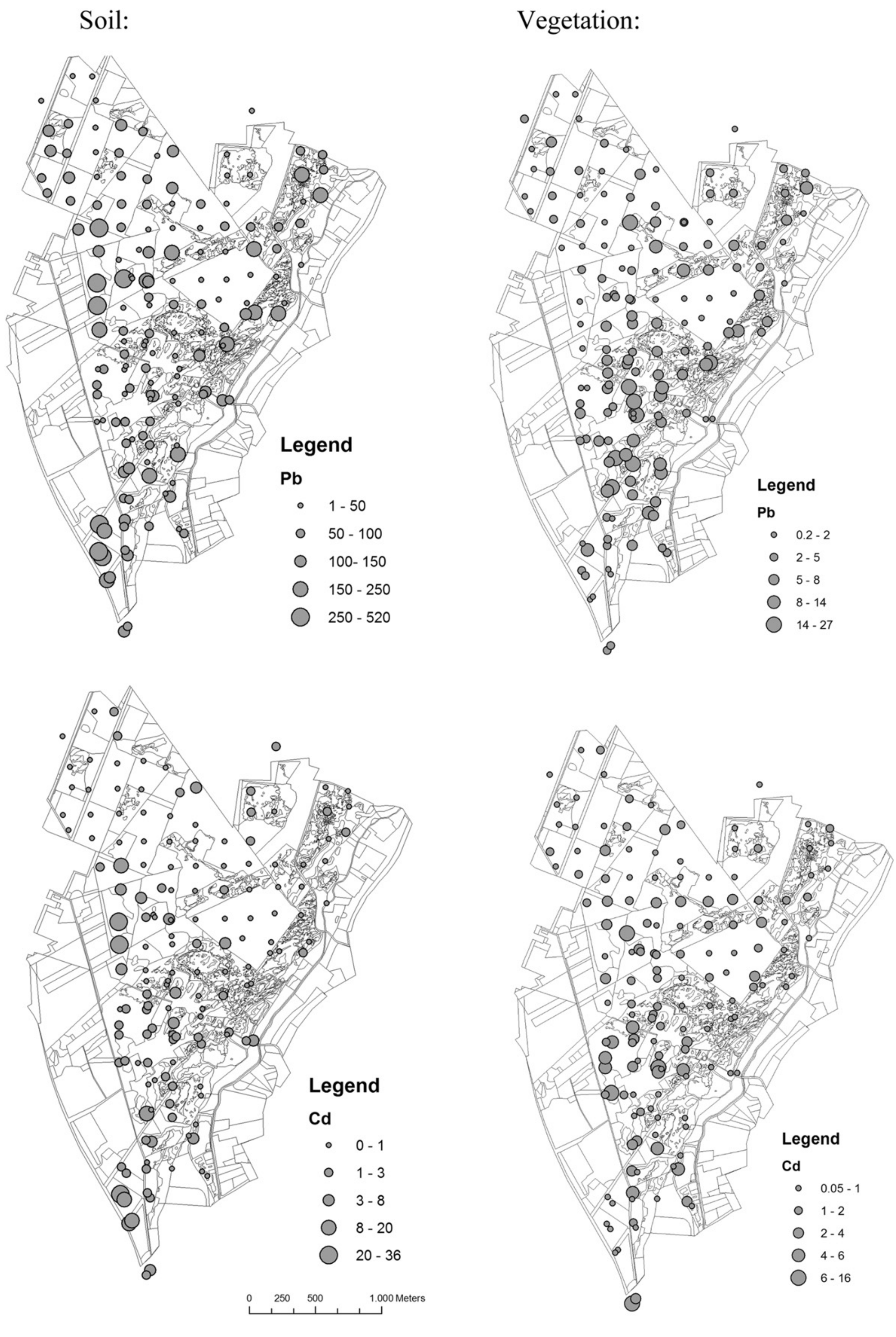

Fig. 2. Spatial differences in Cd and Pb concentrations (μg/g dw) in soil and vegetation (all vegetation types), based on the objective grid of the reserve.

Table 1
Metal specific parameters to describe the relationship between soil and vegetation for different metals, using Equation (1). R^2 is the coefficient of multiple determination and a–e are the partial regression coefficients, $n = 153$, DF = 149.

Metal	a	b	c	d	e	R^2
Cd	0.802	0.252	−0.115	−0.470		0.122
Co	−0.354	0.383	−0.094	−0.563		0.204
Zn	1.948	0.382	−0.074	−0.351		0.298

(Parkpian et al., 2003), Poland (Niesiobedzka, 2012), Nigeria (Ogundiran et al., 2012), Turkey (Cicek et al., 2012) and France (Schreck et al., 2012), the Cd concentrations (0.084–53.1 μg/g dw) were 7–170 times higher, the Zn concentrations (37–3408 μg/g dw) were 15–550 times higher, the As concentrations (0.15–5.05 μg/g dw) were 6 times higher, the Pb (0.63–67.6 μg/g dw) and Cu (1.02–23.9 μg/g dw) concentrations were intermediate and the Co concentrations (0.0002–0.748 μg/g) were 3 times lower than the levels found in those other studies.

On average, Cd concentrations in grass and soft rush exceeded the EC maximum tolerated level (which presents a potential danger to human health, animal health or the environment) of 1 μg/g for plants with a moisture content of 12% (equals 1.14 μg/g dry weight), used for animal feed (2002/32/EC). In heath, soft rush and grass respectively 10%, 25% and 50% of the Pb concentrations exceeded the EC maximum tolerated level of 10 μg/g for plants with a moisture content of 12%, used for animal feed (2002/32/EC). For As only a few soft rush samples exceeded the maximum tolerated level of 4 μg/g for plants with a moisture content of 12%, used for animal feed (2002/32/EC). Several studies already showed that internal metal concentrations in organisms increase with the duration of exposure (Antoniou et al., 1989; Prankel et al., 2005). Therefore the result of this study suggests that especially for the older cows from the reserve, the level of Cd and Pb accumulation might cause health problems such as disruption or damage of the gastrointestinal, renal, nervous or hemopoietic systems (Swarup et al., 2006). Because these non-essential metals antagonize the Zn, Ca, Cu, Mn and Fe metabolism, they also can cause deficiencies of these essential metals. A study on waterbucks with comparable Cd, Pb, Zn and Cu levels in soil and vegetation reported chronic signs of weight loss, poor hair coat, bone and dental deformities and a lower kidney fat index (Jumba et al., 2007).

3.2.2. Seasonal variation and vegetation use by the Galloway cows

During the whole study period, the composition of the 2 herds that were spontaneously formed by the cows, stayed constant. There were no animals switching from one herd to another. The 2 herds turned out to use different parts of the reserve (Fig. 4). Herd 1 was the largest and grazed in the southern part of the reserve. Herd 2 was a bit smaller and used the northern part of the reserve. Because the northern part mostly consisted of forests and the southern part mostly consisted of wetland and pools, it could be expected that this would result in a different vegetation use of the two herds. The statistical analysis to calculate the frequency in which each vegetation type was eaten indeed showed a significant difference between the herds. Herd 2 ate significantly more grass and trees than herd 1. Herd 1 ate significantly more heath and soft rush (Table 2 and Fig. S3).

There was also a significant difference in vegetation use between winter and summer (Table 2). During summer the Galloways almost exclusively consumed grass while in winter they also ate a significant amount of soft rush and heath. This is similar to other studies with large herbivores (Pokorny et al., 2004). The main reason for the seasonal differences is that in winter grass stops growing and grazers have to eat other plants to fulfill their nutritional needs. Also weather conditions can play an important role in the grazing behavior of herbivores. For example: ruminants graze essentially during daylight but when temperature is higher than 25 °C, they adapt their grazing periods to early morning and late evening to avoid the warmest periods (Baumont et al., 2000). This was also observed during the present field study where on a hot day (30 °C) the cows were lying in the shadow of trees instead of grazing during almost the whole observation period. Only for very short periods some cows were grazing in the vicinity of their tree, but never with the whole herd. This way they were grazing on places where they normally didn't. Because of the spatial, temporal, quantitative and qualitative variation of their food, herbivores have to cope with the fluctuating variability of metal levels in their body (Pokorny et al., 2004).

No significant interactions were found between season and herd for the vegetation use of the Galloways. The seasonal differences are probably due to the changes in soil characteristics with changing weather conditions and differences in plant growth and vegetation composition. The differences in vegetation use between the 2 herds are probably due to the fact that they used different types of habitat but this hypothesis needs further investigation. This result suggests that metal exposure of these grazers varies between seasons and between different herds.

3.2.3. Metal exposure of the Galloway cows

Differences in metal exposure of the cows were expected because there was a significant difference in vegetation use between winter and summer and between the two herds and because metal concentrations differed significantly between the vegetation types. Statistical analysis indeed showed significant differences in the mean metal concentrations of total vegetation, between seasons and between the two herds (Fig. 5, Table S4). For all metals except Pb and Cu, the concentration in the vegetation was significantly different between seasons. Except for Co, the mean metal concentrations in the total vegetation also significantly differed between the 2 herds. The exposure to lead was more than twice as high in herd 1 for both seasons. Cadmium exposure was even three times higher for animals of herd 1 during winter. Also for copper, zinc and arsenic herd 1 was exposed to higher concentrations during both summer and winter (Fig. 5). This significant difference in metal exposure between the two herds, living in the same reserve, may be explained by their differences in habitat and vegetation use and the spatial variation in soil metal concentrations. The southern part of the reserve lies closer to the zinc smelter which may lead to higher metal pollution in that part. However, there was not always a clear pollution gradient for all metals (Figs. S1 and S2). Therefore the proportion of the different vegetation types eaten by the cows together with the metal concentrations in those different vegetation types seems the most important parameter to explain this difference in metal exposure. As an example for Cd, Fig. 6 shows the impact of vegetation use on the Cd intake of the Galloways during winter. Although soft rush contributed only for 20–30% to the total diet, it was responsible for 70% of the Cd intake of the cows. For that reason, the proportion of the different vegetation types in the total diet of the cows was used to calculate the mean daily metal intake per herd per season, using Equation (1) (Table 3). For example, for a cow from herd 1, the daily Cd intake during winter was:

$$\mathrm{DI}_{(\mathrm{Cd})} = 12.5\ \mathrm{kg}^*\Big[(0.38^*7.37\ \mu\mathrm{g/g})_{\mathrm{grass}} + (0.29^*0.38\ \mu\mathrm{g/g})_{\mathrm{heath}} + (0.33^*21.19\ \mu\mathrm{g/g})_{\mathrm{soft\ rush}}\Big] = 1238\ 11\ \mu\mathrm{g/day} = 124\ \mathrm{mg\ Cd/day}$$

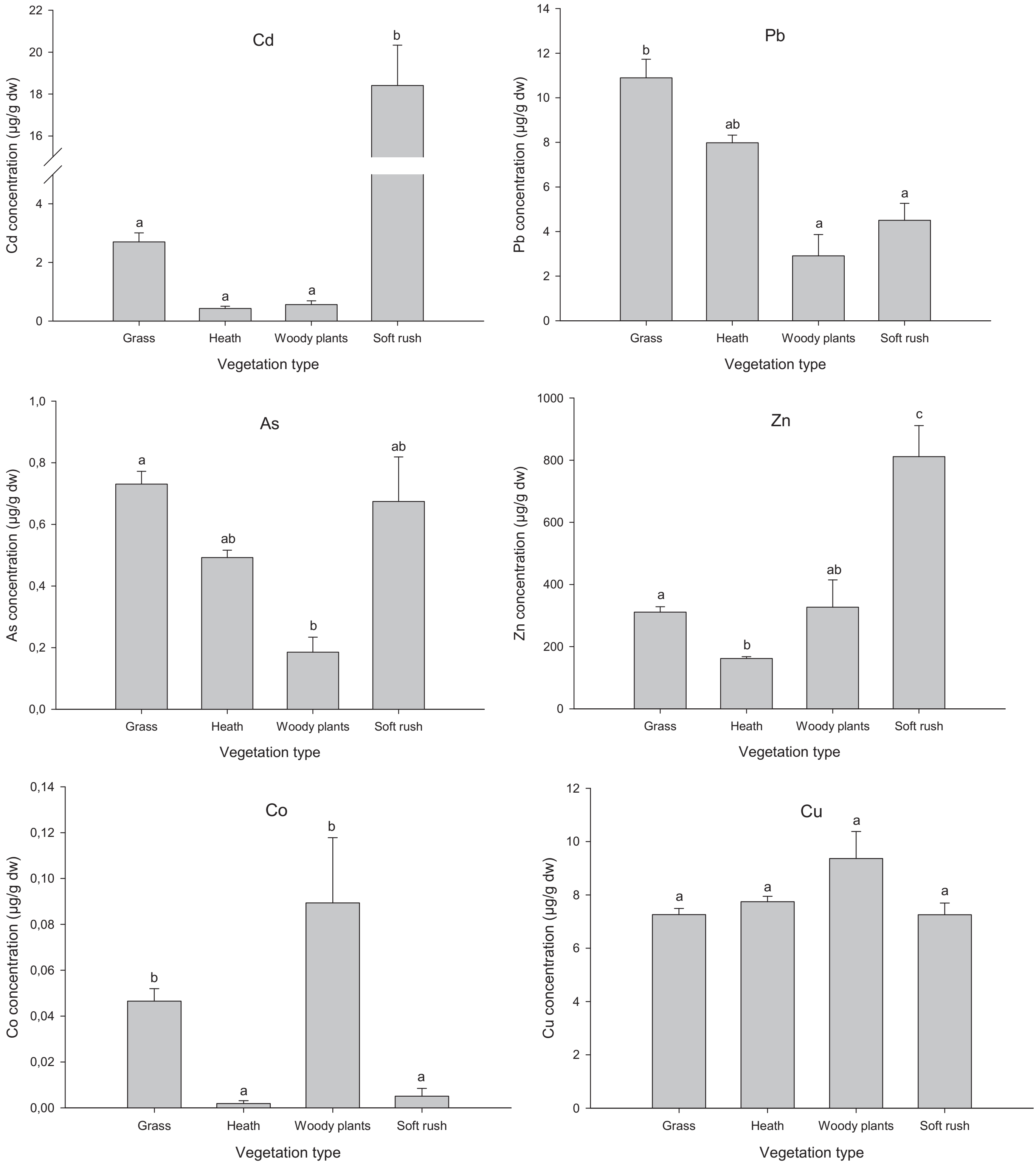

Fig. 3. The mean metal concentrations (dry weight) with their standard errors in the different vegetation types. Vegetation types with the same letter are not significantly different from each other (one way ANOVA with post hoc Tukey–Kramer test).

The calculation of the mean daily Cd intake shows that the high soft rush consumption of herd 1 during winter resulted in a high increase of the mean daily Cd intake (Table 3). Also the Zn concentration was significantly higher in soft rush than in the other vegetation types and this also resulted in a daily Zn intake during winter twice as high as during summer. On the other hand, Pb- and As concentrations were the highest in grass which resulted in an increase of the daily Pb- and As intake during summer, when much more grass was consumed. A similar result was found during a study of Pokorny et al. (2004) where ingestion of fungi during

Table 2
Results of the Fisher's exact test for seasonal variation in vegetation use and variation in vegetation use between the 2 herds.

Vegetation type	Birch %	Oak %	Grass %	Heath %	Soft rush %	Bush %	Fisher's exact test
Winter	2.11	0	42.96	26.76	28.17	0	$p < 0.0001$
Summer	0.74	2.94	94.12	0	0.74	1.47	
Herd 1	0.64	0	59.24	18.47	21.66	0	$p < 0.0001$
Herd 2	2.63	3.51	80.7	5.26	6.14	1.75	

summer–autumn was the most important factor to explain a peak of Hg in the kidneys of roe deer during that period. Also Smith et al., 2010, found that the daily metal intake differs between seasons. Other studies already showed the importance of habitat type and seasonal variation in the availability of vegetation and the associated variation in the diet (Fritsch et al., 2011; Reglero et al., 2008; Vermeulen et al., 2009). The daily Pb and Cd intake was lower than the 0.5 mg Cd/kg body weight per day that causes subclinical effects to sheep and the fatal dosis of 12 mg Pb/kg body weight per day for an adult cow and 6 mg Pb/kg body weight per day for calves (Wilkinson et al., 2003).

3.2.4. Metal concentrations of the Galloways

Fig. 7 and Table S5 summarize the mean metal concentrations in the non-destructive tissues of the Galloways. The mean lead concentration in the blood was 77.6 ± 2.90 μg/l. This concentration is 2–3 times higher than in other studies (Alonso et al., 2000; Smith et al., 2010; Phillips et al., 2011), also higher than the baseline levels

Fig. 4. Map of the Hageven-Plateaux reserve with all vegetation sampling points taken during grazing, showing the differences in habitat use between the 2 Galloway herds.

of 20–60 μg/l for lead in blood of cows but lower than the lowest reported blood lead level of 350 μg/l that has been associated with clinical lead toxicosis in cattle (Ma, 1996). Also for As the mean blood concentration of 8.76 ± 1.74 μg/l was 3 times higher while the mean blood Cd, Cu and Zn concentrations (0.454 ± 0.0231 μg/l, 749 ± 18.3 μg/l and 2550 ± 69.6 μg/l respectively) were comparable to another study with cattle (Alonso et al., 2000). On the other hand, the Cd concentrations were much lower than those found in blood of cows near a steel plant in India (Patra et al., 2006, 2007) or blood from sheep that were exposed to metals during 9 weeks (Phillips et al., 2011).

Metal levels in blood only differed significantly between the two herds for Co and As. Although the calculated exposure between the 2 herds was different for the non-essential (Cd, Pb) and the essential metals (Cu and Zn), this was not reflected in significantly different blood levels.

Metal levels in neck hair differed significantly between the two herds for all metals except Pb (Fig. 7). Most of the metal concentrations were higher in hair of animals from herd 1. This could be expected, knowing that herd 1 was exposed to higher metal concentrations than herd 2, during the whole year. Because coat hair growth in cattle is absent or minimal in winter (Dunnett and Lees, 2003) it can provide information about chronic metal exposure of the animals during last spring and summer. Hair may therefore be a useful tool to detect metal accumulation in grazers especially during these seasons. When the animals reside in the same area during the whole year, as in this case study, coat hair can be useful to measure the general metal accumulation during the previous year, before the hair sample was taken. In this study, the mean Pb concentration in hair was 2–5 times higher while the Cd, Zn and Cu the concentrations were similar to the results of a study of Patrashkov et al. (2003). The mean As-concentration in hair was 10 times higher than found in wool of sheep during a study of Kolacz et al. (1999). The concentration of Pb was similar, Cd was 10 times lower and Zn was 10 times higher than in hair of horses and sheep, suffering from lead poisoning in combination with cadmium, during a study of Liu (2003). The high concentration of lead in the hair of the Galloways in this case study suggests that the animals possibly suffer from lead toxicosis, although this was not suggested by the results of the blood samples. The results indicate that hair is a useful non-destructive monitoring tool for studying the metal exposure of grazers, which is similar to the conclusions of other studies where they even used hair to predict accumulation levels of organs (Beernaert et al., 2007; D'Havé et al., 2006). However, in this case study, hair may be more suitable to use as a monitoring tool for the metal exposure of the whole herd rather than a predictor of the individual metal accumulation levels.

In feces, the mean concentrations of Cd and Pb were respectively 10 and 3 times higher than those found in another study with steers (Gustafson and Olsson, 2004) and with horses although horses have another digestive system (Madejón et al., 2009). For As and Zn the mean concentrations were comparable with those in the study with steers (Gustafson and Olsson, 2004). On the other hand, the Pb concentration was 3 times lower, Zn concentration 2 times higher and Cu concentration was similar than those in sheep feces (Smith et al., 2010). All metal concentrations in feces differed significantly between the two herds (Fig. 7). The differences in metal concentration can again be explained by the differences in metal exposure between the 2 herds. Animals of herd 1 were exposed to higher metal concentrations and this resulted in a higher metal excretion via feces. This suggests that a higher metal exposure not necessarily leads to an increase of the actual assimilation via the intestines which is similar to earlier studies (Lauwerys, 1978; Miller et al., 1967; Tsuchiya, 1979). It is generally assumed that ≤5% of the

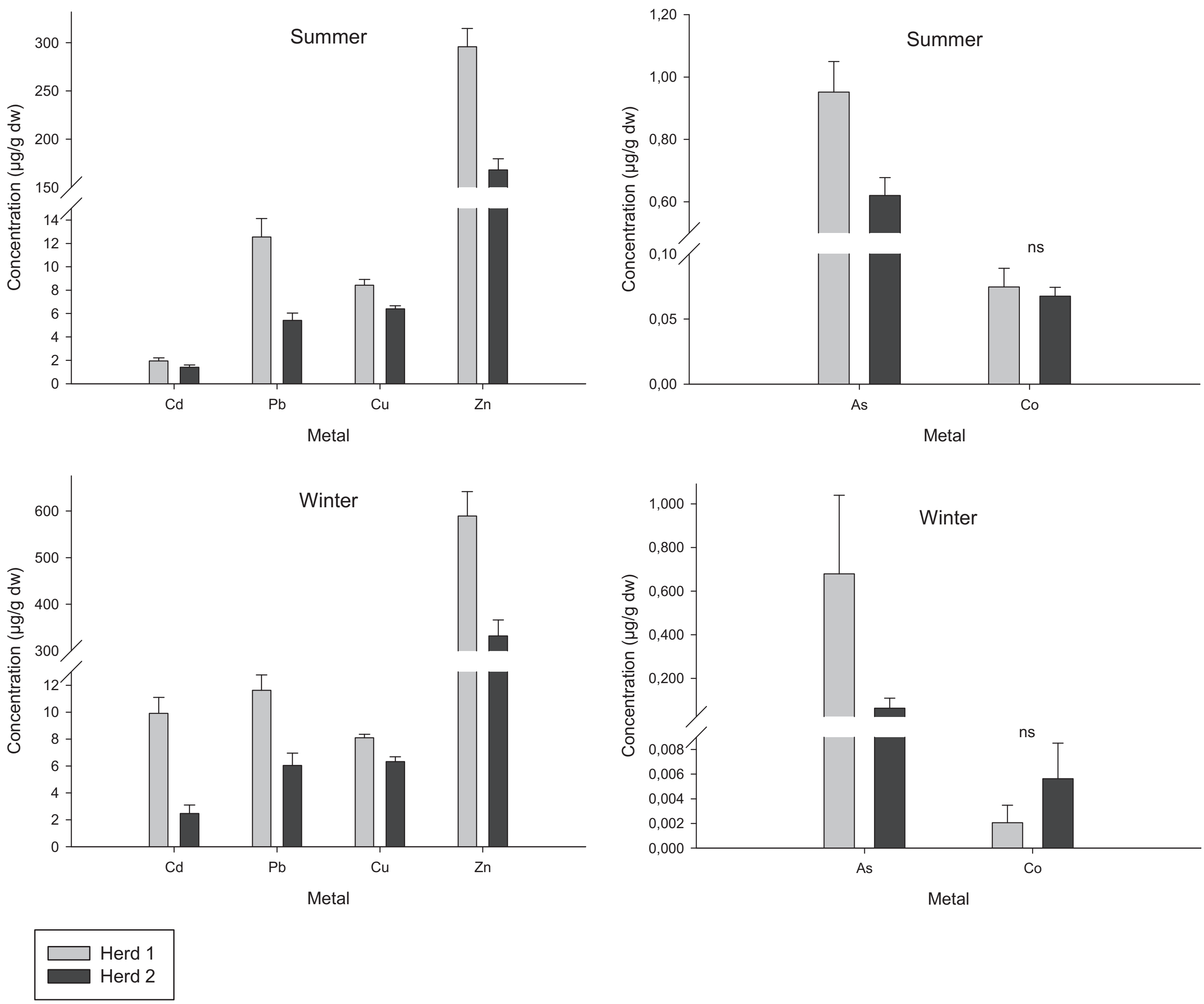

Fig. 5. Mean metal concentrations and standard errors (SE) in total vegetation (all vegetation types), eaten by the two herds during summer and winter. ns: not significantly different.

ingested cadmium and lead concentration is assimilated in adult cows and ≤50% of the ingested lead is assimilated in calves (Wilkinson et al., 2003). In contrast, the absorption of essential metals such as zinc is much higher and can reach up to 80% of the total ingested concentration. There is no effective homeostatic protection against the absorption of non-essential metals such as Pb and Cd but the presence of essential minerals and other nutrients in the food can influence the uptake of several metals (Wilkinson et al., 2003). Lead absorption for example will reduce with higher calcium or phosphate concentrations and will increase with higher protein or fat concentrations in the food (EFSA, 2010). Cadmium absorption will increase with low calcium, iron or protein concentrations (Friberg et al., 1974), but is most commonly affected by the Zn status of the animals (Wilkinson et al., 2003).

When metal concentrations in feces in the present study were compared with their corresponding levels in the ingested vegetation 2 or 3 days earlier, most of them showed a similar pattern in time (Fig. S4). When the concentrations in the eaten vegetation increased, also the concentrations in the feces, collected 2 days later, increased. Nevertheless a correlation analysis ($n = 6$) between the metal concentrations in vegetation and in feces only resulted in a significant positive correlation for Pb. The low number of correlations may be due to the low sample size of 6 for this correlation test. This low sample size was a consequence of the fact that a lot of data points could not be used for this analysis due to the retention time of 2–3 days of food inside the intestines of cattle (Campling et al., 1963; Obitsu et al., 2009; Peyraud et al., 1989; Trinacty et al., 1999) and due to the fact that samples were collected in 2 different herds. The weak correlations between concentrations in feces and vegetation may also be due to variation in vegetation digestibility between the samples, or to the fact that the vegetation in the feces was eaten at another place than the collected vegetation samples, yet on the same day. All fecal metal concentrations were 2–3 times higher than in vegetation (Fig. S4), which is similar to other studies (Madejón et al., 2009). This implies that when feces are used as a biomonitoring tool for environmental metal pollution this factor 2–3 has to be taken into account to avoid overestimation. The results also suggest that the metal concentration in

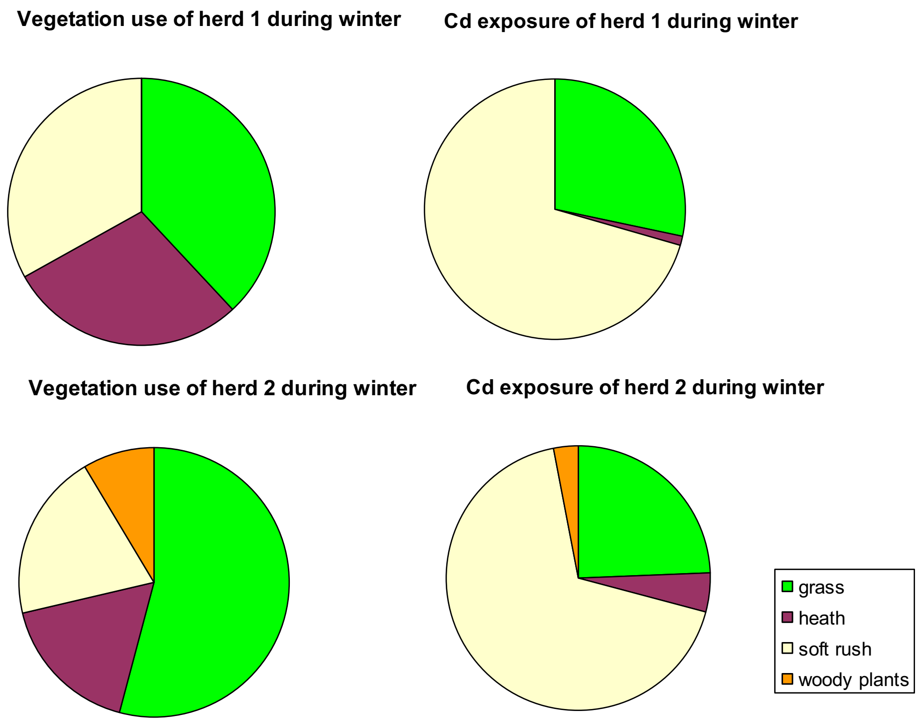

Fig. 6. Percentages of the different vegetation types eaten by the two herds during winter and their corresponding contribution to the Cd exposure of the Galloways.

feces is mostly determined by the metals that have simply passed trough the digestive tract, unabsorbed, and less by the metals that were previously absorbed and metabolized and than subsequently released and excreted in the feces. Therefore the measured metal concentrations are probably the proportion of unabsorbed metals per kg dry weight of the feces. Also the variation in dry weight of the feces due to differences in digestibility of the vegetation has to be taken into account.

Because the metal concentration in feces is the sum of the total metal intake via ingestion of vegetation, soil and drinking water this study also suggests that feces are very useful to measure the total exposure of large herbivores. Soil ingestion differs between herbivore species, vegetation growth and weather conditions (Thornton and Abrahams, 1983). Therefore it is almost impossible to measure the exact amount of ingested soil in the field. Nevertheless soil ingestion is a direct exposure route for most herbivores (Thornton and Abrahams, 1983; Smith et al., 2009). Therefore, using feces has the advantage that the uncertainty about how much soil they ingest while grazing is avoided. In this study direct soil ingestion was not measured or calculated apart from the other parameters. The reason for it was that it could not be measured in the field and because, in contrast with horses and sheep, cows do not eat vegetation very close to the ground. Therefore soil particles adhering to the plants are probably the most important route for soil ingestion by cows. Because the measured vegetation samples in this study were unwashed, metals from adhering soil particles were also included. Some studies reported a soil intake by cattle up to 1.5 kg/day (Wilkinson et al., 2003). It is likely that during the present study direct soil ingestion also contributed to the high metal concentrations in the feces.

Table 3
Mean daily metal intake (mg/day) for a Galloway cow, per herd, per season.

Season	Winter		Summer	
Herd	1	2	1	2
Cd	124	32	24	18
Pb	145	75	157	68
Co	0.1	0.2	0.9	0.9
Cu	101	78	105	80
Zn	7368	4216	3698	2110
As	8	4	12	8

Although no target organs for metal accumulation such as liver or kidney were sampled, the non-destructive tissues might give a good indication of the metal intake level and even of internal contamination. Because no relevant reviews on metal concentrations in vegetation, blood, hair or feces of mammals, and only a few critical values for cows are available, the results of this case study were mostly compared to those of other field studies. In the present study blood did not turn out to be a good tool to measure metal exposure and in other studies blood also did not turn out to be a good predictor for metal accumulation in organs (Maia et al., 2006). Blood metal concentrations generally reflect current or recent exposure and are therefore more useful when the exposure period is rather short (Wittman and Hu, 2002). That is why hair is probably a better tool when chronic exposure is studied. Hair allows retrospective investigation of chronic and past exposure to metals (Pragst and Balikova, 2006). It has been already successfully used as a biomonitoring tool and to a lesser extend as a predictor for internal concentrations in other studies from regions with high metal contamination (D'Havé et al., 2005; Patra et al., 2007; Pereira et al., 2006; Rashed and Soltan, 2005). Feces are the most non-invasive tools and have already been confirmed as a reliable biomonitoring tool for assessing environmental metal pollution, but not as a predictor of metal uptake by organisms (Dauwe et al., 2000; Madejón et al., 2009; Pokorny et al., 2004). In this case study, feces turned out to be a useful tool to predict the total environmental exposure of the grazer. However, more research is necessary to study the exact ratio between oral metal intake and fecal metal concentrations.

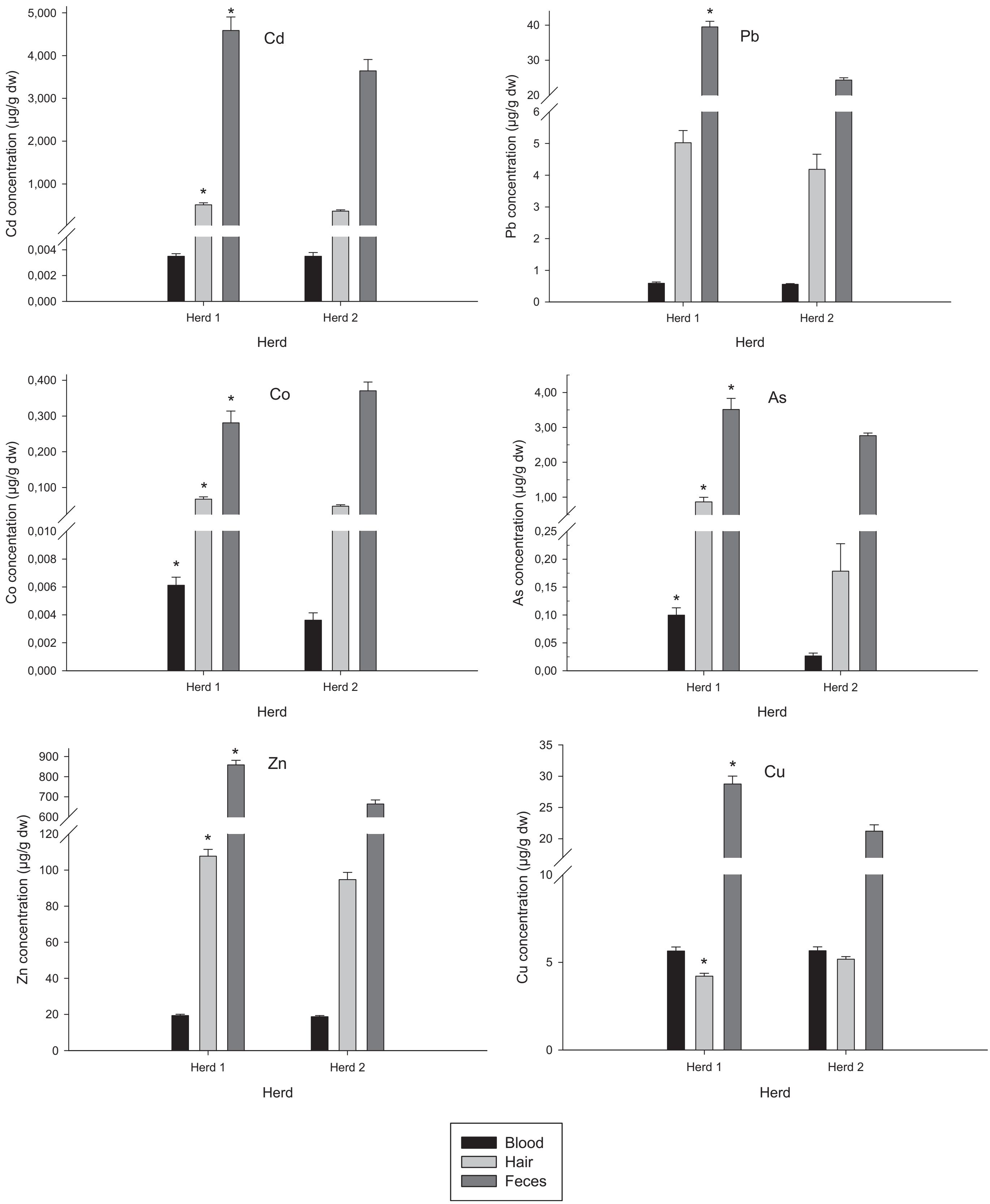

Fig. 7. Mean metal concentrations with their SE in the different tissues of the Galloways (blood, hair and feces). *Significant difference between the 2 herds.

4. Conclusions

This study revealed new insights in the mechanisms of metal accumulation in a terrestrial ecosystem and the exposure of grazers. Spatial heterogeneity has an important influence on the metal uptake of free ranging grazers. In order to predict the exposure and health risks of large grazers it is important to have detailed information of the occurring vegetation types, the spatial habitat use together with the social- and foraging behavior and diet selection of the herbivore species that is studied. In this case study, hair seemed more suitable to monitor the general metal exposure of the whole herd rather than a predictor of the individual metal accumulation levels of the cows. Feces seemed the best tool to measure the general metal exposure via ingestion of forage, soil and water. However, more research is necessary to study the exact ratio between oral metal intake and fecal metal concentrations.

During this case study the differences in diet composition between the two herds seemed the most important parameter to explain the differences in metal exposure of the Galloways. The metal concentrations measured in the diet and the hairs of the cows suggest that for some metals i.e. Pb and Cd, gastrointestinal, renal, nervous or hemopoietic health problems might be possible.

The results of this study are specific for cattle so it can also be useful to conduct this type of study for other herbivore species. The data of this study are useful to validate existing risk assessment models. The results can help to perform more accurate and non-invasive risk assessment studies in the future, also for wildlife. When a risk assessment study is planned for other types of grazers, e.g. horses or sheep, the ingestion of soil and/or plant roots plays probably a more important role in the diet and therefore also in the metal exposure pattern. This has to be taken into consideration.

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Appendix A. Supplementary data

Supplementary data of metal concentrations and results of the statistical analysis can be found online at http://dx.doi.org/10.1016/j.envpol.2012.09.006.

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